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Antibody and T-cell responses during acute and convalescent stages of invasive pneumococcal disease

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SUMMARY

Objective: To understand the pattern of immune responses to pneumococcal proteins during invasive disease as a guide to their development as vaccine candidates.
Methods: The antibody concentration and avidity, as well as frequency of interferon-gamma (IFN-γ)-, interleukin-10 (IL-10)-, and tumor necrosis factor-alpha (TNF-α)-containing CD4+ T-lymphocytes in response to pneumolysin, pneumococcal surface protein A (PspA), and choline-binding protein A (CbpA), during and after invasive pneumococcal disease (IPD) in 20 children were compared to those of 20 healthy matched controls.
Results: During the acute phase of IPD, the concentrations of antibodies against these three pneumococcal proteins were lower, whereas the frequencies of IL-10- and TNF-α-producing CD4+ T-cells were higher, compared to values obtained during convalescence and in healthy controls ($p < 0.01$). In addition, the concentrations of antibodies against the capsular polysaccharides for the serotypes isolated from these patients, were all below the detection level of the assay during both the acute and convalescent phases of IPD.
Conclusion: These data indicate that the recognition of these antigens by the immune system occurs in variable proportions according to the stage of infection, implying the important role of these in the pathogenesis of IPD, and support their usefulness in vaccine development.

1. Introduction

Streptococcus pneumoniae is one of the major causes of respiratory tract infections and invasive diseases in children worldwide.[1,2] Approximately 1 million children under 5 years of age die annually of pneumococcal diseases, and most of these occur in sub-Saharan Africa.[3,4] The high prevalence of invasive disease in developing countries is assumed to be a reflection of the high rate of nasopharyngeal carriage.[5–7] Nasopharyngeal colonization with pneumococci occurs in 20–50% of young children in Western Europe,[8] but can be as high as 90% in children aged <5 years in sub-Saharan Africa.[6,9]

Antibody responses to the polysaccharide antigens play a major role in immunity to pneumococcal diseases. The antibodies induced by pneumococcal conjugate vaccines are effective in protecting against nasopharyngeal carriage and invasive pneumococcal diseases (IPD) by serotypes contained within the vaccine.[10,11] However, the use of conjugate vaccines for protection against IPD is limited not only by their serotype coverage (which varies in different settings), but also by the potential for serotype replacement, and the cost and complexity of production.[12] These issues have led to the consideration of the development of a non-serotype-dependent pneumococcal vaccine.[13] In this regard, pneumococcal proteins and protein–polysaccharide components are promising for use in vaccines, as they have the potential to be inexpensive, immunogenic in young children, and most likely effective against all serotypes. In addition, pneumococcal protein antigens induce T-lymphocyte responses, which contribute to protection against both nasopharyngeal carriage and IPD.[14] Some of the protein candidates being considered include pneumolysin, pneumococcal surface protein A (PspA), and choline-binding protein A (CbpA).[15–17] However, their role in IPD in humans is

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not well understood. Thus, we sought to evaluate both T- and B-cell immune responses to pneumolysin, PspA, and CbpA during and after IPD in order to identify factors contributing to increased vulnerability to IPD, and to determine if the disease process induces changes in the immune responses considered.

2. Methods

2.1. Study site and recruitment of study participants

Subjects were recruited as part of formal surveillance for invasive bacterial disease in children in the Western Region of The Gambia. Acutely ill febrile children aged 0–9 years admitted to the pediatric ward of the Royal Victoria Teaching Hospital, Banjul, The Gambia were investigated by culture of cerebrospinal fluid (CSF) or blood or both. This study was conducted between 2007 and 2009 when the pneumococcal vaccine had not yet been introduced into the national immunization schedule. Those with positive cultures were considered as cases of IPD and were consecutively recruited into this study after written informed consent had been obtained from the parent/caregiver. Healthy children of the same age from a nearby health centre were recruited as controls after parental/caregiver consent was obtained. The study was approved by the joint Gambia Government/MRC Ethics Committee (SCC L2007.53).

Isolation of *S. pneumoniae* from the CSF and venous blood samples was done according to standard procedures, as previously described.[6] Serotype was determined by latex agglutination using serotype-specific antisera, while isolates with equivocal results were confirmed by the Quellung reaction.

2.2. Blood sampling

On admission, 2 ml of venous blood was obtained as soon as IPD was confirmed (acute sample). Appropriate antibiotic and supportive treatments were completed and patients discharged with an instruction to come back to the clinic at 4 weeks after the start of treatment. At this follow-up visit, the child was examined for general well-being as well as for any neurological deficit. Thereafter another venous blood sample (convalescent sample) was obtained. The acute and convalescent blood samples were processed within 6 h of collection. A venous blood sample (2 ml) was obtained from healthy children of the same age attending the well-child clinic at the Sukuta Health Centre (controls).

2.3. Antigens and overnight antigenic stimulation

Recombinant pneumolysin (Ply), CbpA, and PspA were prepared and used as previously described.[18] Pneumococcal culture supernatant was prepared from a standard encapsulated type 2 (D39) *S. pneumoniae* strain (WT). The protein content of the concentrated pneumococcal culture supernatant was determined using the Bio-Rad protein assay (Bio-Rad, UK).

Venous blood samples were collected in heparinized tubes; serum was separated and stored at −70 °C until analysis. The remaining blood sample was resuspended 1:1 in RPMI culture medium, from which 200 μl was taken per stimulation in a polypropylene tube (BD Pharmingen, USA). Blood was cultured overnight with WT (5 μg/ml), Ply (5 μg/ml), phytohemagglutinin (PHA; 5 μg/ml) and purified protein derivative (PPD RT49; SSI, Denmark; 10 μg/ml) were used as positive controls while culture medium alone was the negative control, and processed as previously described .[19] Tubes were vortexed, covered, and maintained at 37 °C, 5% CO_2 overnight (16 h). Brefeldin A (Sigma) was added after 2 h (final concentration 10 μg/ml).

2.4. Whole blood intracellular cytokine staining

Following overnight stimulation, 20 μl of previously titrated surface marker cocktail (CD4-PerCP and CD8-Pacific Blue; both from BD Biosciences, USA) was added to each tube, as previously described.[19] Tubes were then vortexed and incubated for 30 min at room temperature (RT). Two milliliters of FACS lysing solution (BD Pharmingen, USA) was added and the tubes vortexed and incubated for 9 min at RT in the dark, followed by centrifugation for 5 min at 1500 rpm at RT. Following removal of the supernatant, 500 μl of 1× FACS Perm2 solution (BD Pharmingen, USA) was added and the tubes vortexed and incubated for 20 min at RT in the dark. Following centrifugation (1800 rpm, RT, 5 min), the supernatant was carefully removed and 20 μl of cytokine cocktail (interferon-gamma (IFN-γ)–allophycocyanin (APC), tumor necrosis factor-alpha (TNF-α)–fluorescein isothiocyanate (FITC), and interleukin-10 (IL-10)–phycoerythrin (PE); all from BD Pharmingen, USA) was added. Tubes were again vortexed and incubated for 30 min at RT in the dark. Tubes were then washed by adding 1 ml of FACS buffer (phosphate-buffered saline (PBS)/1% fetal calf serum (FCS)/0.02% sodium azide (Az)) and centrifuged at 1800 rpm for 5 min. Finally, the supernatant was removed and cells resuspended in 1% paraformaldehyde (PFA) for acquisition.

2.5. Flow cytometry analysis

All samples were processed with a CyAn ADP™ (Beckman Coulter, USA) flow cytometer following gating on lymphocytes according to 90° forward and side-scatter plots. FACS plots were analyzed using FlowJo software version 6.1.1 (Treestar; Ashland, OR, USA). The proportion of cells producing each cytokine was calculated within the total population of CD4+ or CD8+ T-cells and background subtracted (as determined from the medium-alone negative control).

2.6. Serological determinations

2.6.1. Measurement of anti-pneumococcal IgG

ELISA was used to measure immunoglobulin type G (IgG) antibodies against specific pneumococcal proteins. Microtiter plates (CoStar, USA) were coated overnight at 4 °C with Ply (2 μg/ml), CbpA (5 μg/ml), or PspA (5 μg/ml) in PBS. Plates were then washed with PBS 0.1% Tween 20 (ELISA wash buffer), blocked with 10% fetal calf serum (FCS) in PBS (blocking buffer) for 2 h at RT and washed (two times); serum samples (1/10 and 1/100 dilutions with blocking buffer) were added to the wells. Sandoglobulin (Sandoz UK, kindly provided by David Goldblatt), which contains high IgG antibody titers to Ply, PspA, and CbpA, was used as the standard, starting with a 1/500 dilution that was assigned an antibody titer of 1000 U/ml, for each of the protein antigens. Serum samples and standard were added in duplicate wells and incubated for 90 min on a horizontal shaker (150 rpm) at RT. Following washing, the plates were incubated with alkaline phosphatase-conjugated goat anti-human IgG for 90 min on a horizontal shaker at RT. They were then washed and the color developed by incubating with *p*-nitrophenyl phosphatase in substrate buffer in the dark for 30 min at RT. Absorbance was read at 405 nm on an ELISA reader (Multiscan, Labsystems, Finland) and analyzed using Softmax Pro software (Molecular Devices, Sunnyvale, CA, USA).

The concentrations of IgG antibodies to pneumococcal capsular polysaccharides 5, 6B, and 14 were measured from sera taken during the acute and convalescent phases of IPD, as previously described.[10] The antibody titers to other serotypes isolated from the IPD patients are not routinely done in our laboratory. The titers were expressed in μg/ml after calculation with reference to assigned values of IgG in the 89-SF reference serum. The lower

limit of detection of the assay was 0.001 μg/ml for the serotypes tested, apart from serotype 14 for which it was 0.002 μg/ml.

2.6.2. Measurement of anti-pneumococcal IgG avidity

Plates were coated and blocked as stated above, and each 1/100 dilution of serum and 1/8000 dilutions of standard were added into six duplicate wells and incubated for 2 h on a horizontal shaker (150 rpm) at RT. After washing, sodium thiocyanate in blocking buffer (NaSCN; Sigma, St. Louis, MO, USA) was used to dissociate antibody–antigen complexes. To get a high resolution of antibodies with different avidities, the concentration of NaSCN was titrated. For each sample, control (blocking buffer, 0 M), and a series of five different concentrations of NaSCN (4, 2, 1, 0.5, and 0.1 M solutions) were added into a pair of wells containing the samples or standard for 15 min, and immediately washed four times with the wash buffer. Thereafter, the remaining steps of the procedure were essentially as done for antibody measurement above. As a measure of the avidity of IgD antibodies present in the serum samples, the avidity index (AI) was used. AI was calculated as the percentage of the antibodies that remained bound after NaSCN treatment. It was calculated by dividing the endpoint titer of the serum sample with NaSCN treatment by the end-point titer of the sample without NaSCN treatment multiplied by 100. Reproducibility of the method was ensured by including a control serum on each plate.

2.7. Statistical analysis

Comparisons of cases (acute or convalescent stages) to healthy controls were conducted using the Wilcoxon rank sum test, due to non-normal distribution of the data. Comparisons of acute versus convalescent data were carried out using paired *t*-tests if the distribution of differences was normal or the Wilcoxon matched pairs signed rank test if the distribution of differences was non-normal. Analyses were done using STATA release 10 statistical software (Stata Corp., College Station, TX, USA).

3. Results

Twenty children, 14 boys and 6 girls, were identified as having IPD within the study period. *S. pneumoniae* was isolated from the CSF in all of them, and also from blood in four of them. Ten of the *S. pneumoniae* isolates were subjected to serotyping, which showed that five were serotype 14, while one each were serotypes 5, 6B, 9 V, 10A, and 35B (Table 1). One child was HIV-1-seropositive. Their age ranged from 1 month to 6 years. Four children (all with meningitis alone) died during the hospital admission and another five subjects did not come for the follow-up visit. Therefore nine cases did not have convalescent blood samples available for analysis. There was an equal ratio of boys to girls in the control group and these were similar to the cases in age and ethnicity, as well as place of domicile.

3.1. Serological responses

During the acute phase of IPD the median (interquartile range (IQR)) antibody concentrations (U/ml) were: 199 (145–306) to Ply,

Table 1
Pneumococcal anti-capsular antibody titers to the serotypes isolated from children with invasive pneumococcal disease (IPD)[a]

Patient ID	Serotype isolated	Acute serotype anti-capsular antibody (μg/ml)	Convalescent serotype anti-capsular antibody (μg/ml)
PC-040	10A	ND	ND
PC-042	14	<0.002	0.001
PC-048	14	<0.002	NA
PC-054	5	<0.001	0.001
PC-076	35B	ND	ND
PC-077	14	<0.002	0.001
PC-080	6B	<0.001	NA
PC-087	14	<0.002	NA
PC-088	14	<0.002	NA
PC-096	9V	0.07	NA

ND, not done; NA, not available.

[a] Pneumococcal anti-capsular antibody titers in serum against the serotypes isolated from each patient during acute and convalescent phases of IPD. Antibody against the capsular antigen to the serotype isolated from the CSF of these patients was measured in the corresponding serum obtained during the acute and convalescent phases of IPD as previously described. The titers are expressed in μg/ml after calculation with reference to assigned values of IgG in the 89-SF reference serum. The lower limit of detection of the assay was 0.001 μg/ml for the serotypes tested, apart from serotype 14 for which it was 0.002 μg/ml.

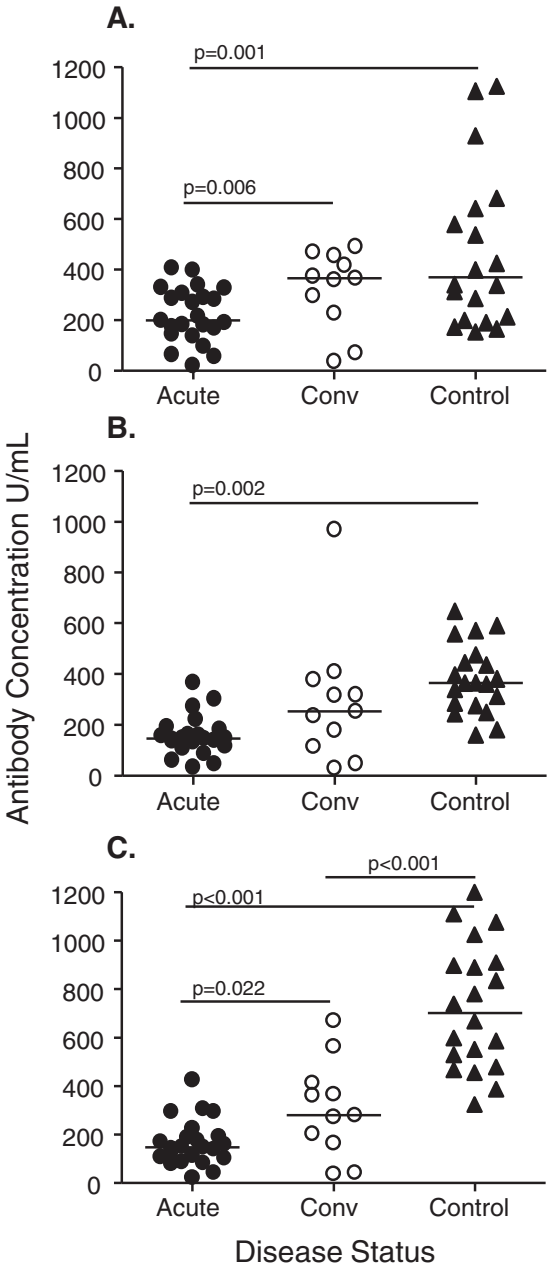

Figure 1. Comparison of antibody levels during acute and convalescent phases of invasive pneumococcal disease (IPD). Antibody against pneumolysin (Ply) (A), pneumococcal surface protein A (PspA) (B), and choline-binding protein A (CbpA) (C) was assessed by ELISA in subjects with acute IPD ($n = 20$) and compared to the same subjects in the convalescent phase (Conv; $n = 11$) and healthy controls ($n = 20$). The horizontal lines indicate the median and the *p*-values indicate significant differences between the groups.

146 (113–188) to PspA, and 147 (102–191) to CbpA; these were significantly lower compared to the healthy controls (Ply 369 (206–662), PspA 366 (280–460), and CbpA 703 (504–904)) (Figure 1). The median (IQR) antibody concentrations during the convalescent phase were: 366 (227–455) to Ply, 253 (115–378) to PspA, and 279 (164–413) to CbpA; these were also generally higher compared to the values obtained in the acute phase, with those of Ply and CbpA being significantly higher (p = 0.006 and 0.022, respectively; Figure 1, A and C). The median antibody concentrations in the convalescent phase of the disease still tended to be lower than the concentrations observed in the healthy control children, but only that of CbpA was significantly lower ($p < 0.001$; Figure 1C). The anti-capsular antibody levels to the specific serotype isolated from each patient were below the detection limits for the assay, and consequently below the protective level of 0.35 μg/ml, in both the acute and convalescent phases of the disease (Table 1).

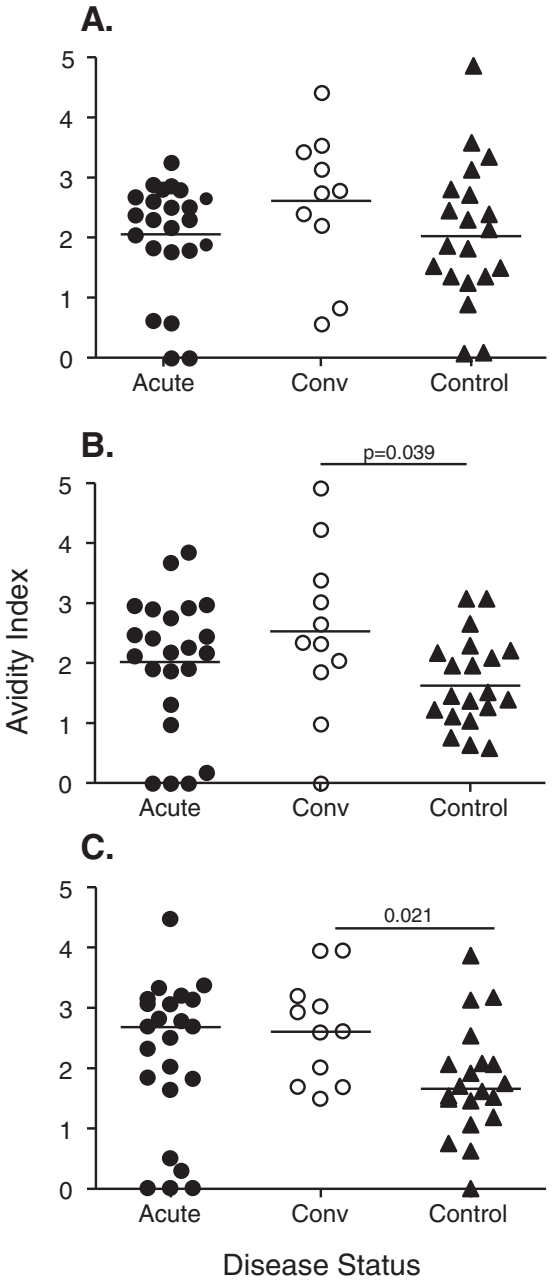

Figure 2. Comparison of antibody avidity indices during acute and convalescent phases of invasive pneumococcal disease (IPD). The avidity index of antibody to pneumolysin (Ply) (A), pneumococcal surface protein A (PspA) (B), and choline-binding protein A (CbpA) (C) was assessed by ELISA in subjects with acute IPD (n = 20) and compared to the same subjects in the convalescent phase (Conv; n = 11) and healthy controls (n = 20). The horizontal lines indicate medians and the p-values indicate significant differences between the groups.

The avidity of the antibodies to the three pneumococcal proteins during the acute phase was lower than that in the convalescent phase of the disease, but the differences were not statistically significant (Figure 2). Unlike the antibody concentrations measured during the acute and convalescent phases of the disease, the antibody avidity in the convalescent phase was significantly higher than that of the control group for PspA (p = 0.039) and CbpA (p = 0.021), but not for Ply (Figure 2).

3.2. T-cell responses during and after IPD were mainly from CD4+ cells

The cellular immune responses were evaluated by measuring the frequency of CD4+ and CD8+ T-cells that expressed IFN-γ, IL-10, or TNF-α, by flow cytometry following overnight stimulation with pneumococcal antigens. Following lymphocyte gating, cells were then analyzed for percent CD4+ and CD8+ cells expressing any of the three cytokines (Figure 3A). We noted that the responses were mainly from CD4+ T-cells in both the acute and convalescent phases of the illness, but there was very low production in the acute phase (Figure 3B, top panel), which was restored to all antigens in the convalescent phase (Figure 3B, bottom panel). There was no significant difference in the frequency of IFN-γ-expressing CD4+ T-cells between the acute and convalescent phases following stimulation with both pneumococcal and control antigens (Figure 4, A, D, and G). The median (IQR) percentage of IL-10-expressing CD4+ T-cells in the acute phase following stimulation with WT (0.3 (0.27–0.70)) and Ply (0.28 (0.19–0.385)), were significantly higher compared to the frequencies observed in the healthy control children: 0.12 (0.06–0.20) and 0.13 (0.05–0.23), respectively (p = 0.001 for both; Figure 4, B and E). The frequencies were also significantly higher during the acute phase compared to the convalescent phase with 0.11 (0.03–0.31) and 0.12 (0.06–0.23) percentage of CD4+ IL-10+ cells in response to WT and Ply stimulation, respectively (p = 0.014 and 0.012, respectively; Figure 4, B and E). The IL-10 levels were comparable to those seen in the healthy controls in the convalescent phase (Figure 4, B and E). Similarly, the median proportion of CD4+ T-cells expressing TNF-α during the acute phase was significantly higher compared to the convalescent phase after stimulation with Ply (0.24 (0.06–0.52) vs. 0.08 (0.026–0.26); p = 0.015) and WT (0.4 (0.28–1.35) vs. 0.07 (0.01–0.44); p = 0.01) (Figure 4, C and F). Proportions of CD4+ TNF-α+ cells during the acute phase were also significantly higher than in the healthy controls (p = 0.001 for both; Figure 4, C and F). In contrast, there was no difference in the frequency of CD4+ T-cells expressing any of these three cytokines in response to PHA stimulation for any of the groups (Figure 4, G, H, and I).

4. Discussion

Our data show that Ply, PspA, and CbpA pneumococcal proteins induce antibody and T-cell responses in children in The Gambia. The concentrations of antibodies to the pneumococcal proteins evaluated were low during the acute phase of IPD as compared to the levels during convalescence and in healthy controls. These antibody levels increased during the convalescent phase, but were still somewhat lower than in the healthy controls. In contrast to the antibody levels, the avidity was higher during the acute and convalescent phases as compared to the controls, particularly for PspA and CbpA. The acute phase of the disease was characterized by significantly higher frequencies of IL-10- and TNF-α-producing

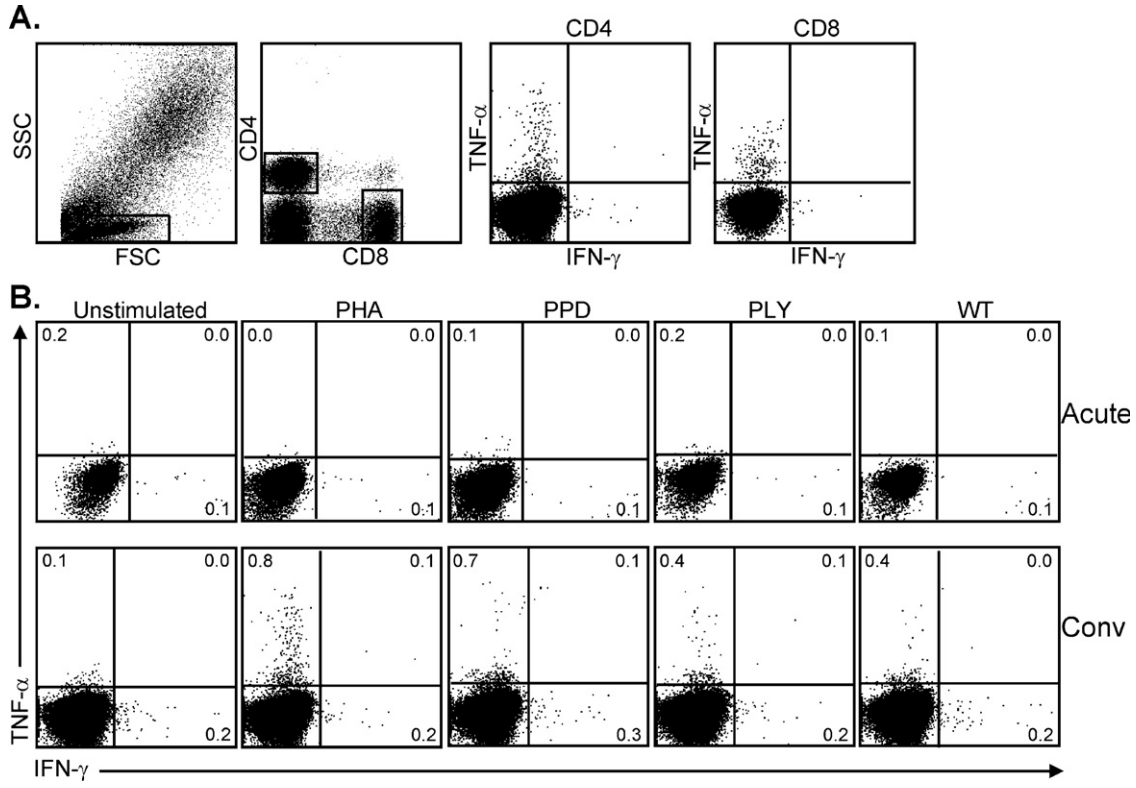

Figure 3. Intracellular cytokine analysis during acute and convalescent phases of invasive pneumococcal disease (IPD). Representative flow cytometry dot plots showing that responses to pneumococcal antigens were mainly from CD4+ T-cells (A). CD4+ responses to phytohemagglutinin (PHA), purified protein derivative (PPD), pneumolysin (Ply), and standard encapsulated type 2 (D39) *S. pneumoniae* strain (WT) antigens were determined following overnight stimulation of whole blood. Representative flow cytometry dot plots show very low responses to all antigens in the acute phase of the infection and restoration during the convalescent (Conv) phase.

CD4+ T-cells, which returned to levels similar to those of the healthy controls in the convalescent phase.

We have demonstrated that the antibody concentration to the three pneumococcal proteins was lower during the acute phase of IPD as compared to the convalescent phase and in healthy controls. The serotype anti-capsular polysaccharide antibody titers were also below the detection limit of the assay and the protective level for invasive disease. The host defense against *S. pneumoniae* depends largely on the binding of antibodies and complement to the surfaces of the pathogen.[20,21] Although the protective titers to pneumococcal proteins are yet to be determined, our data support the requirement of antibodies to these pneumococcal proteins for protection against IPD. While antibody titers are indicators of the magnitude of response, avidity is an index of functionality, and increases with secondary exposure to an antigen.[22,23] Avidity indices at low antibody titers are usually unreliable, such as those measured during the acute phase of IPD when the antibody titers were low. However, the difference in avidity observed between the convalescent phase and the healthy controls with comparable antibody titers is reliable. Thus, our finding of relatively higher antibody avidity to these proteins after IPD compared to healthy controls suggests a response to exposure to the pneumococcus, and further underlines the fact that the absolute concentration of the antibody is important to curtail the infection. Similarly, high antibody levels to Ply and CbpA have been shown to protect against nasopharyngeal carriage of pneumococcus and probably IPD.[11] A study in Finnish children also showed that lower antibody titers to PspA predisposed to IPD.[24] Subjects colonized by *S. pneumoniae* have also been observed to have higher serum IgG concentrations to Ply compared to patients with pneumococcal bacteremia.[25] The persistently low antibody concentration during convalescence compared to the healthy controls could also suggest the existence of an intrinsic defect in the production of antibodies to these proteins. It is expected that large amounts of pneumococcal protein antigens expressed in the host during IPD would induce significantly higher antibody responses as compared to healthy controls whose antibodies are most likely produced following colonization by pneumococcus in the nasopharynx.[26] Thus the relatively lower concentrations during convalescence, particularly in the face of increasing avidity, might suggest impaired response in antibody production to these proteins following invasive pneumococcal disease. Conversely, since increased antibody levels and avidity are indicative of B-cell memory response to an antigen, our data could also imply a consumption of the antibody in the formation of antibody–antigen complexes at the sites of infection. Also, an inadequate antibody response might be related to the ability of high doses of pneumococcal polysaccharides to depress the immune system as well as deplete the memory pool. The relationship between IL-10 and antibody production may be complicated.[27] IL-10 has been known to promote humoral immune responses. High levels of IL-10 have been associated with a suppression of the antibody response to pneumococcus, which could explain the low antibodies observed during the acute phase of IPD, when the frequency of IL-10-expressing CD4+ T-cells was high as compared to healthy controls observed in our study.[28]

Data in mice indicate that CD4+ T-cell interactions with pneumococci are crucial in providing protection in the host.[14] Further evidence for the role of CD4+ T-cells comes from the increased risk of IPD in HIV-positive individuals with low CD4+ T-cell counts.[29] Our data show no variation in IFN-γ production following stimulation with pneumococcal antigens during the acute or convalescent phases, suggesting a defect in the IFN-γ response could have rendered them vulnerable to invasive pneumococcal disease, as previously described.[30,31] The frequency of T-cell responses expressing cytokines to most antigens including

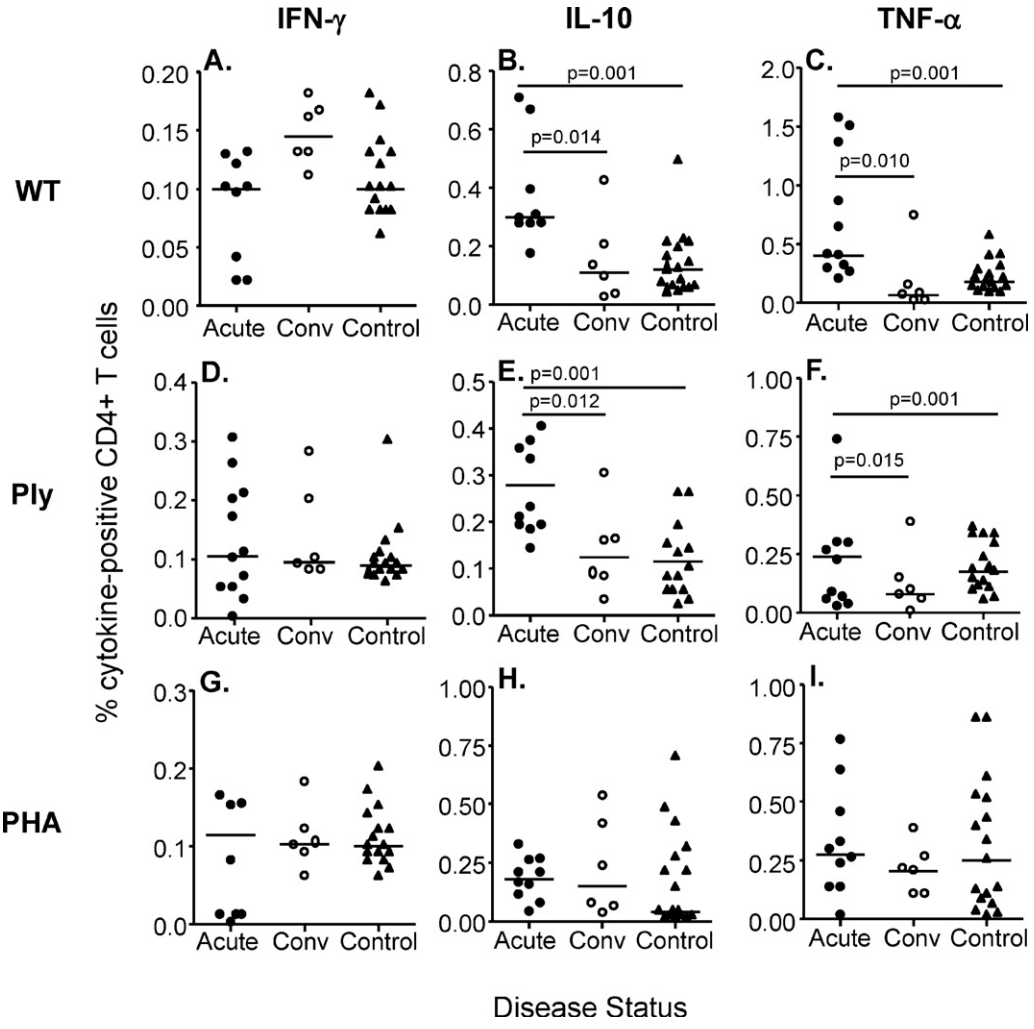

Figure 4. CD4+ production of interferon-gamma (IFN-γ), interleukin-10 (IL-10), and tumor necrosis factor-alpha (TNF-α) at different stages of invasive pneumococcal disease (IPD). The frequency (% CD4+ T-cells) of IFN-γ-, IL-10-, and TNF-α-producing CD4+ T-cells was measured following overnight stimulation with either (D39) *Streptococcus pneumoniae* strain (WT), recombinant pneumolysin (Ply), or phytohemagglutinin (PHA). The horizontal lines indicate the medians of n = 11 for acute phase, n = 6 for convalescent (Conv) phase, and n = 16 for healthy controls; p-values indicate significant differences between any of the two groups designated.

PHA were generally low, as is usually observed, when compared to adults.[32,33] However, the lack of an IFN-γ response to a non-pneumococcal antigen such as PHA is an indication of a generalized limitation of the IFN-γ that could either be intrinsic to the host or from reciprocal inhibition of the high IL-10 response.[34,35] The role of TNF-α in IPD is controversial. Our data show increased TNF-α production during the acute phase that returns to normal during convalescence, in keeping with its inflammatory role.

To the best of our knowledge, this study is the first to measure T-cell and antibody responses to pneumococcal proteins in association with anti-capsular antibodies during IPD in children. We have demonstrated low antibody concentrations and variations in CD4+ T-cell immune responses to pneumococcal protein antigens; these do not provide conclusive evidence of their protective role against IPD, as they might either be a cause or consequence of the disease. Nevertheless, our data do indicate that these antigens are expressed at sufficient levels to be relevant to the immune system, which warrants their further assessment as potential vaccine candidates.

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Conflict of interest: The authors declare that there are no financial or personal relationships with other people or organizations that have inappropriately influenced this work.

References

1. Mulholland K. Magnitude of the problem of childhood pneumonia. *Lancet* 1999;**354**:590–2.
2. Siber GR. Pneumococcal disease: prospects for a new generation of vaccines. *Science* 1994;**265**:1385–7.
3. Mulholland EK, Adegbola RA. Bacterial infections—a major cause of death among children in Africa. *N Engl J Med* 2005;**352**:75–7.
4. Obaro S, Adegbola R. The pneumococcus: carriage, disease and conjugate vaccines. *J Med Microbiol* 2002;**51**:98–104.

5. Faden H, Duffy L, Wasielewski R, Wolf J, Krystofik D, Tung Y. Relationship between nasopharyngeal colonization and the development of otitis media in children. Tonawanda/Williamsville Pediatrics. *J Infect Dis* 1997;**175**:1440–5.
6. Hill PC, Akisanya A, Sankareh K, Cheung YB, Saaka M, Lahai G, et al. Nasopharyngeal carriage of *Streptococcus pneumoniae* in Gambian villagers. *Clin Infect Dis* 2006;**43**:673–9.
7. Gray BM, Converse 3rd GM, Dillon Jr HC. Epidemiologic studies of *Streptococcus pneumoniae* in infants: acquisition, carriage, and infection during the first 24 months of life. *J Infect Dis* 1980;**142**:923–33.
8. Aniansson G, Alm B, Andersson B, Larsson P, Nylen O, Peterson H, et al. Nasopharyngeal colonization during the first year of life. *J Infect Dis* 1992;**165**(Suppl 1):S38–42.
9. Hill PC, Cheung YB, Akisanya A, Sankareh K, Lahai G, Greenwood BM, et al. Nasopharyngeal carriage of *Streptococcus pneumoniae* in Gambian infants: a longitudinal study. *Clin Infect Dis* 2008;**46**:807–14.
10. Saaka M, Okoko BJ, Kohberger RC, Jaffar S, Enwere G, Biney EE, et al. Immunogenicity and serotype-specific efficacy of a 9-valent pneumococcal conjugate vaccine (PCV-9) determined during an efficacy trial in The Gambia. *Vaccine* 2008;**26**:3719–26.
11. Zhang Q, Bernatoniene J, Bagrade L, Pollard AJ, Mitchell TJ, Paton JC, et al. Serum and mucosal antibody responses to pneumococcal protein antigens in children: relationships with carriage status. *Eur J Immunol* 2006;**36**:46–57.
12. Choo S, Finn A. New pneumococcal vaccines for children. *Arch Dis Child* 2001;**84**:289–94.
13. Bernatoniene J, Finn A. Advances in pneumococcal vaccines: advantages for infants and children. *Drugs* 2005;**65**:229–55.
14. Kadioglu A, Coward W, Colston MJ, Hewitt CR, Andrew PW. CD4-T-lymphocyte interactions with pneumolysin and pneumococci suggest a crucial protective role in the host response to pneumococcal infection. *Infect Immun* 2004;**72**:2689–97.
15. Morrison KE, Lake D, Crook J, Carlone GM, Ades E, Facklam R, et al. Confirmation of psaA in all 90 serotypes of *Streptococcus pneumoniae* by PCR and potential of this assay for identification and diagnosis. *J Clin Microbiol* 2000;**38**:434–7.
16. Paton JC. Novel pneumococcal surface proteins: role in virulence and vaccine potential. *Trends Microbiol* 1998;**6**:85–7. discussion 87–8.
17. Rosenow C, Ryan P, Weiser JN, Johnson S, Fontan P, Ortqvist A, et al. Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of *Streptococcus pneumoniae*. *Mol Microbiol* 1997;**25**:819–29.
18. Mureithi MW, Finn A, Ota MO, Zhang Q, Davenport V, Mitchell TJ, et al. T cell memory response to pneumococcal protein antigens in an area of high pneumococcal carriage and disease. *J Infect Dis* 2009;**200**:783–93.
19. Sutherland JS, Young JM, Peterson KL, Sanneh B, Whittle HC, Rowland-Jones SL, et al. Polyfunctional CD4(+) and CD8(+) T cell responses to tuberculosis antigens in HIV-1-infected patients before and after anti-retroviral treatment. *J Immunol* 2010;**184**:6537–44.
20. Brown EJ, Hosea SW, Hammer CH, Burch CG, Frank MM. A quantitative analysis of the interactions of antipneumococcal antibody and complement in experimental pneumococcal bacteremia. *J Clin Invest* 1982;**69**:85–98.
21. Brown EJ, Hosea SW, Frank MM. The role of antibody and complement in the reticuloendothelial clearance of pneumococci from the bloodstream. *Rev Infect Dis* 1983;**5**(Suppl 4):S797–805.
22. Usinger WR, Lucas AH. Avidity as a determinant of the protective efficacy of human antibodies to pneumococcal capsular polysaccharides. *Infect Immun* 1999;**67**:2366–70.
23. Ekstrom N, Ahman H, Verho J, Jokinen J, Vakevainen M, Kilpi T, et al. Kinetics and avidity of antibodies evoked by heptavalent pneumococcal conjugate vaccines PncCRM and PncOMPC in the Finnish Otitis Media Vaccine Trial. *Infect Immun* 2005;**73**:369–77.
24. Virolainen A, Russell W, Crain MJ, Rapola S, Kayhty H, Briles DE. Human antibodies to pneumococcal surface protein A in health and disease. *Pediatr Infect Dis J* 2000;**19**:134–8.
25. Musher DM, Phan HM, Baughn RE. Protection against bacteremic pneumococcal infection by antibody to pneumolysin. *J Infect Dis* 2001;**183**:827–30.
26. Simell B, Korkeila M, Pursiainen H, Kilpi TM, Kayhty H. Pneumococcal carriage and otitis media induce salivary antibodies to pneumococcal surface adhesin a, pneumolysin, and pneumococcal surface protein a in children. *J Infect Dis* 2001;**183**:887–96.
27. Zhang Q, Bagrade L, Clarke E, Paton JC, Nunez DA, Finn A. Bacterial lipoproteins differentially regulate human primary and memory CD4+ T and B cell responses to pneumococcal protein antigens through Toll-like receptor 2. *J Infect Dis* 2010;**201**:1753–63.
28. Khan AQ, Shen Y, Wu ZQ, Wynn TA, Snapper CM. Endogenous pro- and anti-inflammatory cytokines differentially regulate an in vivo humoral response to *Streptococcus pneumoniae*. *Infect Immun* 2002;**70**:749–61.
29. Kyaw MH, Rose Jr CE, Fry AM, Singleton JA, Moore Z, Zell ER, et al. The influence of chronic illnesses on the incidence of invasive pneumococcal disease in adults. *J Infect Dis* 2005;**192**:377–86.
30. Rubins JB, Pomeroy C. Role of gamma interferon in the pathogenesis of bacteremic pneumococcal pneumonia. *Infect Immun* 1997;**65**:2975–7.
31. Sun K, Salmon SL, Lotz SA, Metzger DW. Interleukin-12 promotes gamma interferon-dependent neutrophil recruitment in the lung and improves protection against respiratory *Streptococcus pneumoniae* infection. *Infect Immun* 2007;**75**:1196–202.
32. Ota MO, Vekemans J, Schlegel-Haueter SE, Fielding K, Whittle H, Lambert PH, et al. Hepatitis B immunisation induces higher antibody and memory Th2 responses in new-borns than in adults. *Vaccine* 2004;**22**:511–9.
33. Vekemans J, Amedei A, Ota MO, D'Elios MM, Goetghebuer T, Ismaili J, et al. Neonatal bacillus Calmette–Guerin vaccination induces adult-like IFN-gamma production by CD4+ T lymphocytes. *Eur J Immunol* 2001;**31**:1531–5.
34. Zhang Q, Bernatoniene J, Bagrade L, Paton PC, Mitchell TJ, Hammerschmidt H, et al. Regulation of production of mucosal antibody to pneumococcal protein antigens by T-cell-derived gamma interferon and interleukin-10 in children. *Infect Immun* 2006;**74**:4735–43.
35. O'Garra A, Vieira P. T(H)1 cells control themselves by producing interleukin-10. *Nat Rev Immunol* 2007;**7**:425–8.